

# Increased levels of miR-124 in human dental pulp stem cells alter the expression of neural markers

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## ABSTRACT

Auditory neuropathy is the particular form of deafness in humans which cannot be treated by replacement therapy. Human dental pulp stem cells (hDPSCs) are derived from an ectomesenchymal neural crest cell population. Therefore, they possess a promising capacity for neuronal differentiation and repair. miR-124, a key regulator of neuronal development in the inner ear, is expressed at high levels in auditory and vestibular neurons. Here, we evaluated the possible effect of miR-124 in alteration of neural protein markers expression. Using quantitative reverse transcription-PCR (qRT-PCR) analyses and immunofluorescence staining, we studied the expression patterns of neural progenitor markers (Nestin, NOTCH1, and SOX2) and neural markers ( $\beta$ -tubulin III, GATA-3, and peripherin) upon transfection of hDPSCs with miR-124. The qRT-PCR results showed that Nestin was upregulated 6 h post-transfection. In contrast, Nestin expression exhibited a decreasing trend 24 h and 48 h post-transfection. Higher levels of  $\beta$ -tubulin III, 6 h and 16 h post transfection in RNA level as compared with control cells, were determined in transfected DPSCs. However,  $\beta$ -tubulin-III expression decreased 48 h post-transfection. The immunofluorescence results indicated that transfection of hDPSCs with miR-124, only affected Nestin among the studied neural progenitor and neural marker expression in protein level.

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## 1. Introduction

Sensorineural hearing loss commonly known as irreversible damage to hair cells or auditory nerves. Cochlear implants or hearing aids can improve hearing disability caused by lack of auditory hair cells. However, the effectiveness of an implant greatly depends on appropriate functioning of auditory nerves (Geleoc and Holt, 2014). Auditory neuropathy (i.e., loss or degeneration of spiral ganglion neurons [SGNs]) is considered a serious problem because

there is no replacement therapy. In this regard, cell therapy has opened up promising horizons for the treatment and replacement of SGNs (Rivolta, 2010). In recent decades, using a variety of stem cell types, researchers have made progress toward the development of auditory nerve-like neurons and neural progenitor cells (Mahmoudian-Sani et al., 2017; Mohammad-Reza et al., 2017; Rivolta, 2010). An important step toward establishing and developing cell-based therapeutics for auditory system is to select an efficient strategy and appropriate cell population to differentiate into target cells that can then be used for corrective purposes (Rivolta, 2010). Attempts have been made previously to differentiate SGNs using growth factors and neurotrophic agents, including brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), sonic hedgehog (SHH), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF), or alteration of the expression levels of transcription factors, including NGN1 (Chen et al., 2012; Reyes et al., 2008). Also, a number of investigations have examined the possible aspects of miRNAs in inner ear development (Reza and Ghahfarrokhi, 2017). miRNAs have been described as a class of noncoding small RNAs that regulate expression of genes through

**Abbreviations:** human dental pulp stem cells, hDPSCs; quantitative reverse transcription-PCR, qRT-PCR; spiral ganglion neurons, SGNs; brain derived neurotrophic factor, BDNF; neurotrophin-3, NT3; sonic hedgehog, SHH; basic fibroblast growth factor, bFGF; epidermal growth factor, EGF; bovin serum albumin, BSA; bone morphogenetic protein 4, BMP4.

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degradation of mRNA or inhibition of translation after transcription (Mahmoodian sani, Hashemzadeh-Chaleshtori, Saidijam, Jami and Ghasemi-Dehkordi, 2016; Maiorano and Mallamaci, 2010). Increasing evidence suggests that miRNAs play pivotal regulatory roles in eukaryotic gene expression including transcriptional and post-transcriptional levels (Ambasudhan et al., 2011). Among miRNAs, various studies demonstrated high expression levels of miR-124, which is known to induce neurogenesis, in auditory and vestibular neurons (Elkan-Miller et al., 2011a; Maiorano and Mallamaci, 2010; Michael D. Weston, Marsha L. Pierce, Sonia Rocha-Sanchez, Kirk W. Beisel, 2006). Embryonic stem cells (Gunewardene et al., 2014; Reza and Ghahfarrokhi, 2017; Shi et al., 2007), induced pluripotent stem cells (iPSCs) (Gunewardene et al., 2014; Nishimura et al., 2009), and adult stem cells, including neuronal (Parker et al., 2007; Wei et al., 2008), bone marrow-derived (Kondo et al., 2005), and inner ear stem cells (Chen et al., 2012), obtained from human and animal cell sources have been used for many purposes (Ghasemi-Dehkordi et al., 2015) including *in vitro* differentiation of auditory neurons. The different tissue types can be used to isolate the stem cells (Alge et al., 2010). In recent years, researches have proposed that dental pulp, a soft part in the pulp cavity of the tooth, may provide a ready source of stem cells (d'Aquino et al., 2009). Human dental pulp stem cells (hDPSCs) are derived from an ectomesenchymal population of neural crest stem cells. Therefore, they possess a promising capacity for neuronal differentiation and repair (Gronthos et al., 2000). As the proportion, availability, and proliferation rate of DPSCs surpass those of bone marrow stromal cells (Kim et al., 2012), DPSCs have been used recently for regenerative medicine purposes (Arthur et al., 2008; Tatullo et al., 2015). DPSCs express pluripotency markers, including SSEA-4, NANOG, and OCT-4 (Tatullo et al., 2015), and they can differentiate into a neuronal lineage (Janebodini et al., 2011; Takeyasu, M., T. Nozaki, 2006; Tatullo et al., 2015). The moral issues connected with using embryonic stem cells, together with the fact that ectomesenchymal DPSCs can be extracted from adults, make DPSCs very promising for neuro-regeneration purposes. Here, we evaluated the possible roles of miR-124 in differentiation of DPSCs toward SGNs. With this aim in mind, we transfected miR-124 into hDPSCs and then evaluated the expression of neural progenitor markers (Nestin, SOX2, and NOTCH1) and neural markers (GATA-3,  $\beta$ -tubulin III, and peripherin).

## 2. Materials and methods

### 2.1. Cell culturing and transfection of hDPSCs with miR-124

Oligonucleotide sequences for the miR-124 mimic and scrambled negative control miRNA were obtained from Exiqon (Qiagen, Denmark). DPSCs were donated by the Royan Institute for Biotechnology (Isfahan, Iran). Approximately  $15 \times 10^4$  DPSCs were cultured in six-well plates with high glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS and 1% Penicillin-Streptomycin. When the cells reached 70–80% confluency (after 24 h), transfection complexes were prepared (Invitrogen, Massachusetts). Briefly, either miRNA mimics or scrambled miRNA were mixed with Lipofectamine 2000 reagent (Invitrogen, MA), and the mixtures was then separately transfected in a final concentration of 24 nM (selected following the literature search) into cells in antibiotic and FBS-free high-glucose DMEM. After 4 h of transfection, the transfection medium was replaced with high-glucose DMEM and 10% FBS containing Penicillin-Streptomycin (1%). The cells were then incubated for an additional 16–48 h.

### 2.2. RNA isolation, cDNA synthesis, and qRT-PCR

Total RNA was extracted from the samples (6 h, 16 h, 24 h, and 48 h post transfection) using Trizol reagent (Sigma, MI) and quantified using a NanoDrop spectrophotometer (Thermoscientific, MA). The transfection efficiency of the miR-124 mimics was evaluated using BONmiR (Stem Cell Technology Research Center, Tehran, Iran) detection kits, including a BON-miR miRNA 1st-Strand cDNA Synthesis Kit and BON-miR High Specificity miRNA QPCR Core Reagent Kit. U47 was used as an endogenous control gene in the qRT-PCR. Equal amounts of total isolated RNA (1  $\mu$ g) per sample were reverse transcribed using a cDNA synthesis kit (Yektatajhi-zazma [YTA], Tehran, Iran) and were transferred into the qRT-PCR reaction. The transcription levels of Nestin,  $\beta$ -tubulin III, GATA-3, and peripherin were evaluated using transcript specific primers and SYBR<sup>®</sup> Green PCR Master Mix (Yektatajhi-zazma [YTA], Tehran, Iran). Specific cycling parameters in the qRT-PCR included an initial denaturation step at 95 °C for 3 min, denaturation at 95 °C for 15 s, annealing at 58 °C for Nestin and  $\beta$ -tubulin III, annealing at 60 °C for GATA-3, and annealing at 62 °C for peripherin (all for 20s), followed by an extension step at 72 °C for 25 s. The number of cycles was optimized at 40. The primer sequences used were as follows: Nestin, (forward) CACCCCTCAGCCCTGACCAC and (reverse) CCTCTATGGCTGTTCTTCTCTACCA;  $\beta$ -tubulin III, (forward) CTCAGGGGCTTTGGACATC and (reverse) CAGGCAGTCGCAGTTTTCAC; GATA-3, (forward) GACGGTCAAGGCAACCACG and (reverse) CCAGGGTAGGGATCCATGAAGC; peripherin, (forward) CACGCTCTCATTTGGCTCTTC and (reverse) GGCTCTCGTCTCAGATTCTC; and 18s rRNA, (forward) GTAACCCGTTGAACCCATTCGT and (reverse) ACCATCCAATCGGTAGTAGCGACG. The transcription level of 18s rRNA was used as an endogenous control. The  $2^{-\Delta\Delta Ct}$  analysis algorithm was used to determine the relative quantification of each sample at the time points analyzed. qRT-PCR reactions were run using a Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia).

### 2.3. Immunostaining

To assess the expression of neural progenitor protein markers (Nestin, NOTCH1, and SOX2), both miR-124 transfected and control cells were fixed in 4% paraformaldehyde in PBS at room temperature and washed three times with washing buffer (1% bovin serum albumin (BSA) in PBS containing 10% normal donkey serum and 0.3% Triton X-100 (Sigma, Missouri). Immunostaining was carried out using a standard protocol and a Human/Mouse/Rat Neural Progenitor Cell Marker Antibody Panel, according to the manufacturer's instructions (R&D Systems, Canada). Cell nuclei were stained with DAPI. The cells were exposed to primary antibodies (mouse monoclonal anti-Nestin IgG<sub>2A</sub>, goat anti-Notch IgG, or goat anti-SOX2 IgG) at a final concentration of 10  $\mu$ g/ml. After three wash steps, secondary antibodies, including donkey anti-mouse IgG (NorthernLights<sup>™</sup> NL493-conjugated antibody or donkey anti-goat NL493-conjugated secondary antibody (R&D Systems, Canada), were applied at a 1:200 dilution rate in PBS, supplemented with 1% BSA. Similarly, immunostaining for GATA-3 (ab199428), peripherin (ab106276), and  $\beta$ -tubulin III (ab78078) (Abcam, Cambridge, U.K.) expression was carried out using the standard protocol of the manufacture. In all the experiments, the technical negative controls excluded the primary antibody.

### 2.4. Statistical analysis

Statistical analyses of the qRT-PCR data were performed using GraphPad statistical software (GraphPad Software, CA). The relative quantification data were analyzed using the Mann–Whitney test.

### 3. Results

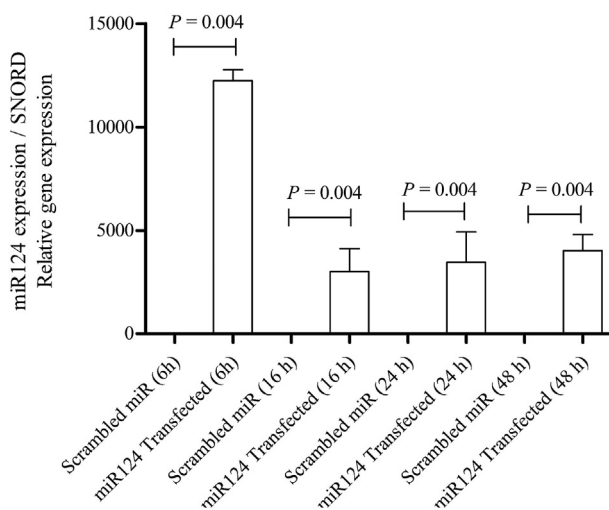
#### 3.1. The pattern of post-transfection of miR-124 levels

According to the characterization data on the DPSCs provided by the Royan Institute for Biotechnology (Isfahan, Iran), these cells exhibit high expression of STRO-1, CD146, CD73, CD90, and CD105 (data not shown). They are also positive for collagen type-I but negative for collagen type-III and CD45. DPSCs have been shown to have ability to differentiation into adipogenic, osteogenic, and chondrogenic lineages. In the present study, miR-124 was transfected into DPSCs using standard methods. To evaluate the level of miR-124 at different times post-transfection, miR-124 levels were analyzed by qRT-PCR. The results of the qRT-PCR analysis showed that the miR-124 level gradually decreased 16 h, 24 h, and 48 h post-transfection (Fig. 1), possibly due to degradation of miR-124.

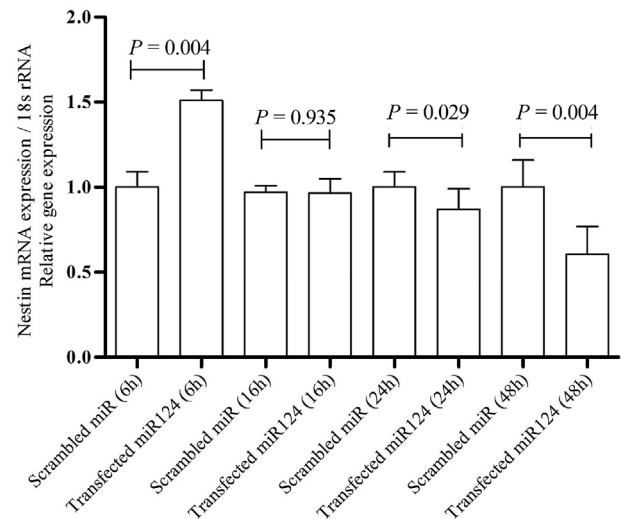
#### 3.2. An increased level of miR-124 6 h post-transfection affected the expression of nestin but not that of NOTCH1 or SOX2

Nestin is an intermediate filament protein, which is a characteristic element of neural stem/progenitor cells. This protein is consistently expressed during differentiation toward neural precursor lineages (Lendahl et al., 1990; Mignone et al., 2004). Six hours post transfection, the qRT-PCR analyses demonstrated an increased level of Nestin mRNA ( $P = 0.004$ ) in transfected DPSCs under all the conditions tested (Fig. 2). However, Nestin expression exhibited a decreasing trend 24 h and 48 h post-transfection (Fig. 2). Upon normalization with the median expression value, the median fold change in Nestin after 6 h, 16 h, 24 h, and 48 h was 1.51, 0.99,  $-0.87$ , and  $-0.60$ , respectively (Livak, methods, & 2001, n.d.).

The immunofluorescence analysis revealed that Nestin was upregulated upon hDPSC transfection with miR-124 (Fig. 3A). In addition to Nestin, we examined the protein expression of SOX2 and NOTCH1 in transfected hDPSCs. The SOX2 protein is an important transcription factor belonging to the SRY-related HMG box family that is encoded in neural progenitors and is implicated in neural commitment and self-renewal (Ferri et al., 2004; Neves et al., 2007; Pevny and Nicolis, 2010). NOTCH1 is a member of the Notch family, which plays a role in developmental processes by



**Fig. 1.** qRT-PCR analysis of the miR-124 level in DPSCs after transfection (6 h, 16 h, 24 h, and 48 h). As shown by the data, the highest increase in the miR-124 level occurred 6 h post-transfection as compared with control cells (Mann-Whitney,  $P = 0.004$ ). The data were normalized to expression levels in the control.



**Fig. 2.** qRT-PCR analysis of Nestin mRNA expression in DPSCs post-transfection. As shown by the data, Nestin mRNA expression was upregulated 6 h post-transfection as compared with that of the control (Mann-Whitney,  $P = 0.004$ ) and downregulated 24 h (Mann-Whitney,  $P = 0.029$ ) and 48 h (Mann-Whitney,  $P = 0.004$ ) post-transfection. No statistically significant alteration in Nestin mRNA expression was observed 16 h post-transfection. The data were normalized to expression levels in the control.

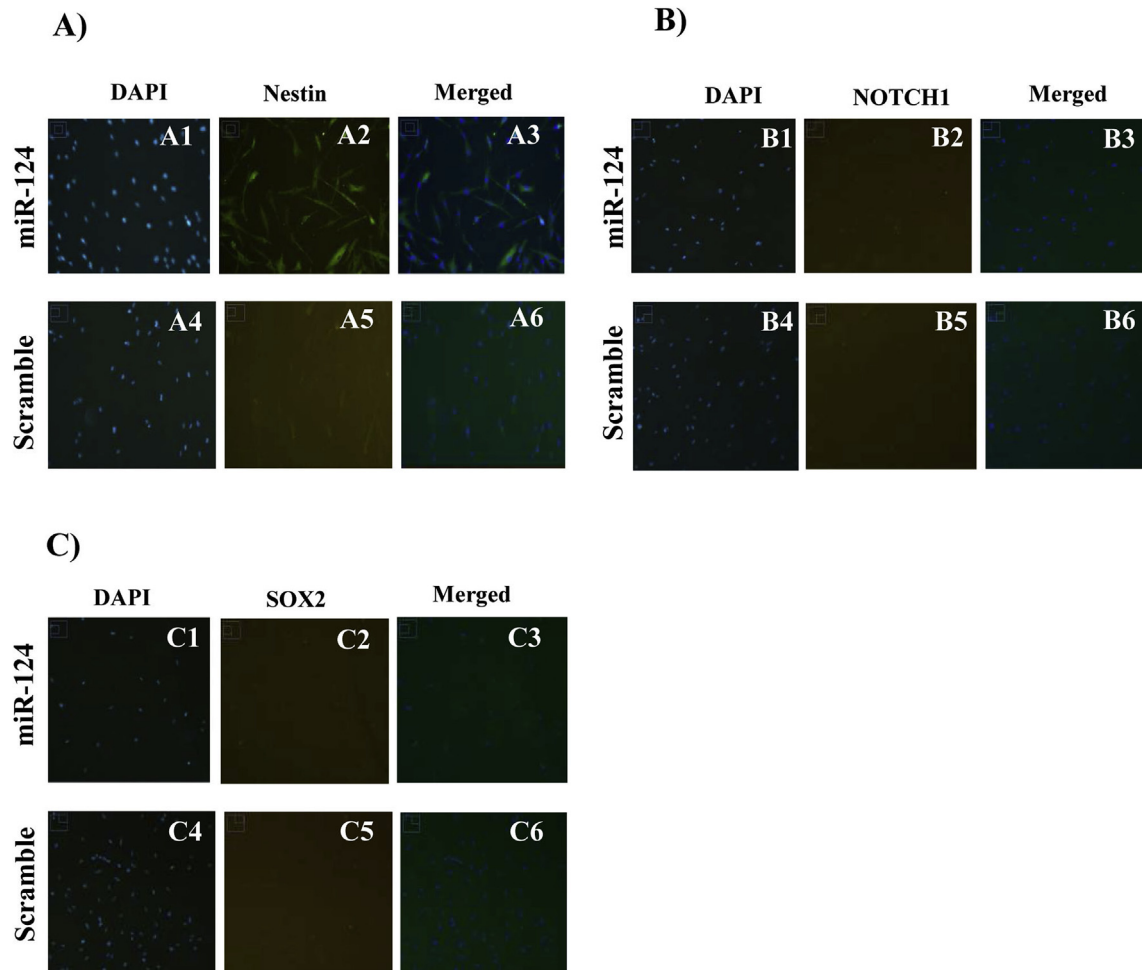
controlling cell fate decisions (Woo et al., 2009). NOTCH1 is expressed in developing sensory epithelia of the mammalian inner ear (Liu et al., 2012). The immunofluorescence analysis showed that unlike Nestin, there was no significant alteration in NOTCH1 (Fig. 3B) or SOX2 expression post-transfection with miR-124 (Fig. 3C).

#### 3.3. An increased level of miR-124 affected the expression of $\beta$ -tubulin III but not that of GATA-3 and peripherin

$\beta$ -tubulin III is a microtubule member of the tubulin family of neural-specific markers (Caccamo et al., 1989; Sullivan and Cleveland, 1986). The results revealed a temporal alteration in the expression of the neuron-specific marker  $\beta$ -tubulin III at the mRNA level. DPSCs transfected with miR-124 mimics had higher levels of this marker 6 h ( $P = 0.005$ ) and 16 h ( $P = 0.008$ ) post-transfection as compared with those in control cells. However, the expression of  $\beta$ -tubulin III decreased 48 h ( $P = 0.005$ ) post-transfection (Fig. 4A). Upon normalization with the median expression value, the median  $\beta$ -tubulin III expression fold change 6 h, 16 h, 24 h, and 48 h post-transfection was 1.50, 1.21, 1, and  $-0.5$  respectively. However, the immunofluorescence analysis revealed no significant alteration in  $\beta$ -tubulin III expression upon hDPSC transfection with miR-124 6 h post-transfection (Fig. 5A). GATA-3, as a transcription factor, has an essential role in auditory development (Lawoko-Kerali et al., 2004) and peripherin (type III intermediate neurofilament), known as a marker for mature type II primary auditory neurons (Hafidi, 1998; Lawoko-Kerali et al., 2004). The results of qRT-PCR and immunofluorescence analyses revealed no significant qualitative alteration in mRNA (Fig. 4B and C) and protein (Fig. 5B and C) expression levels of GATA-3 and peripherin in the miR-124 transfected samples.

### 4. Discussion

Hearing loss is a frequent form of sensory deficit and represents a severe social and health problem in humans (Ciorba et al., 2012). Furthermore, loss of auditory nerves can influence the efficacy of

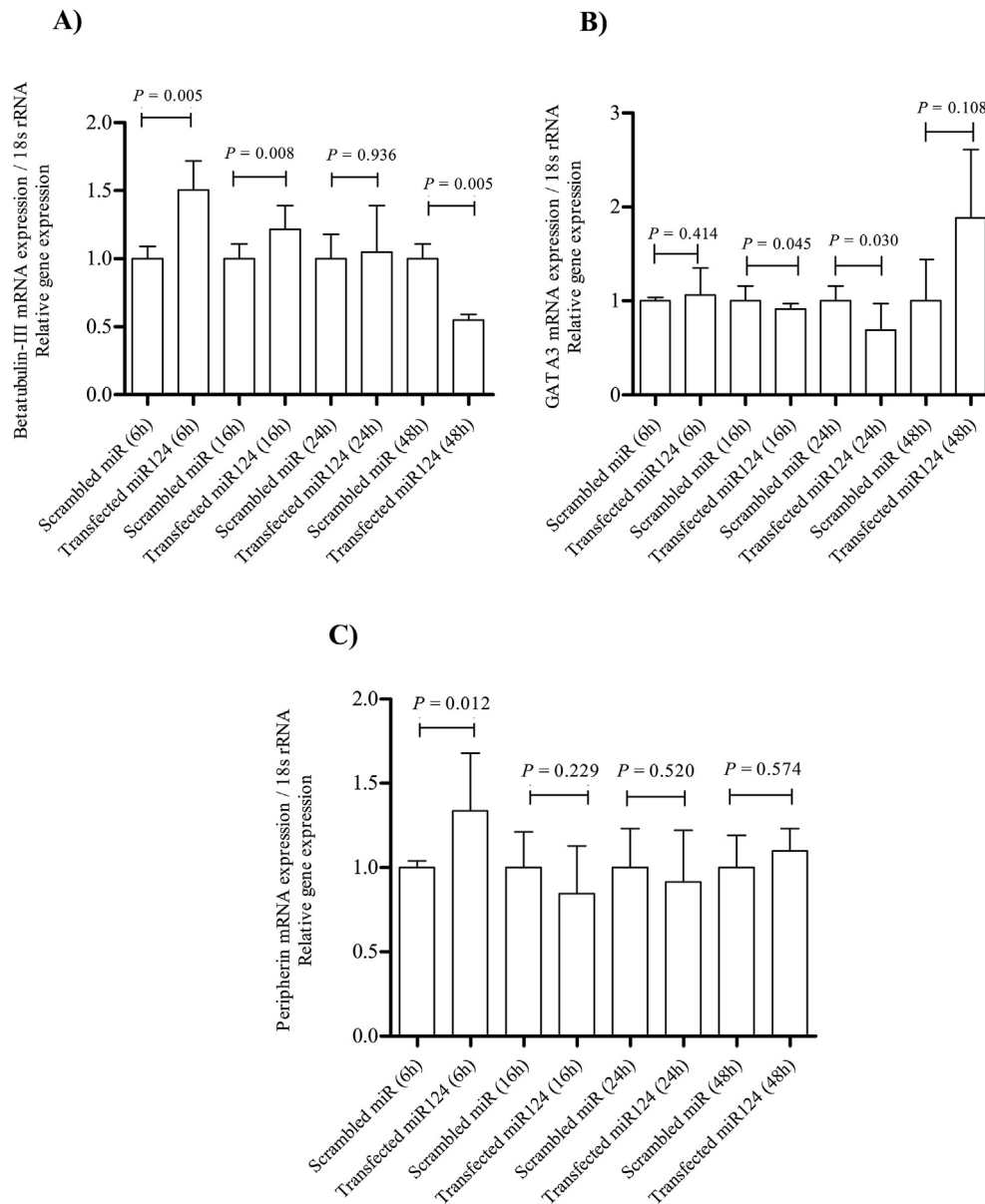


**Fig. 3.** Immunofluorescence analysis of Nestin (A), NOTCH1 (B), and SOX2 (C) expression in DPSCs 6 h after transfection with miR-124 (A1,2,3; B1,2,3; C1,2,3) versus scrambled miR (A4,5,6; B4,5,6; C4,5,6). As shown by these figures, an increased level of miR-124 6 h post-transfection affected the expression of Nestin but not that of NOTCH1 or SOX2.

cochlear prostheses (Géléoc and Holt, 2014; Müller and Barr-Gillespie, 2015). The present study is the first to reveal that temporal increases in the level of miR-124 mimic in DPSCs affected the expression of some neural progenitors and neural markers. The importance of miR-124 in neurogenesis, particularly in SGNs development in the inner ear has been well documented (Elkan-Miller et al., 2011a). This study aimed to investigate aspects of DPSC differentiation toward SGNs upon transfection with miR-124. The main reason underlying the selection of DPSCs in the present study is the neural crest origin of these cells. Thus, they offer excellent potential as regards differentiation toward neural progenitor cells and neurons. However, the potential of DPSC differentiation toward auditory neurons has not been studied. Attempts have been made in recent years to generate SGNs using various protocols and cell sources (Chen et al., 2012; Shi and Edge, 2013). The strategies and approaches included co-culturing, application of growth and neurotrophic factors, genetic manipulation, and induction of transcription factors inside stem cells to increase the expression of neuron-related markers (Chen et al., 2009, 2012; Reyes et al., 2008; Rivolta et al., 2006; Shi et al., 2007). Previous research also demonstrated that neural progenitors derived from human embryonic stem cells differentiated into auditory neurons. Shi et al. (2007) demonstrated BMP4 treatment can lead to the upregulation of NGN1, Brn3, TrkB, and TrkC, pointing to sensory neuron generation. Using human fetal auditory stem cells, Chen

et al. evaluated the function and expression of stem cell markers (NGN1, Neurofilament 200, Parvalbumin, and  $\beta$ -tubulin III) in differentiated cells (Chen et al., 2009). In a murine model, embryonic stem cells expressing NGN1 were grown in medium containing BDNF and Glial cell-derived neurotrophic factor (GDNF). The results revealed that 50–75% of embryonic stem cells expressed markers of early neurons and that the greater part of the cells were glutamatergic phenotype (Reyes et al., 2008). In another promising study, researchers generated active neurosensory progenitors using media containing EGF, bFGF, and small peptide Y27632 (Needham et al., 2014). Another strategy employed for the generation of SGNs was co-culturing of iPSCs with mouse cochlea (Nishimura et al., 2009). Gunewardene et al. (2014) established an *in vitro* neural induction protocol for differentiation of human iPSCs toward neurosensory cells. Their results showed that this protocol was capable of generating functionally active neurosensory progenitors. Using various differentiation protocols, Chen et al. acquired two types of otic progenitors and electrophysiologically evaluated the functional properties of the resulting cells. They demonstrated that these otic progenitors differentiated into auditory neurons following transplantation into a murine model of neuropathic deafness (Chen et al., 2012). Other researchers demonstrated using SHH and retinoic acid aided the induction of glutamatergic sensory neuron markers, pointing to the differentiation of stem cells toward sensory cells (Kondo et al., 2005). In a murine model, Jiang et al.

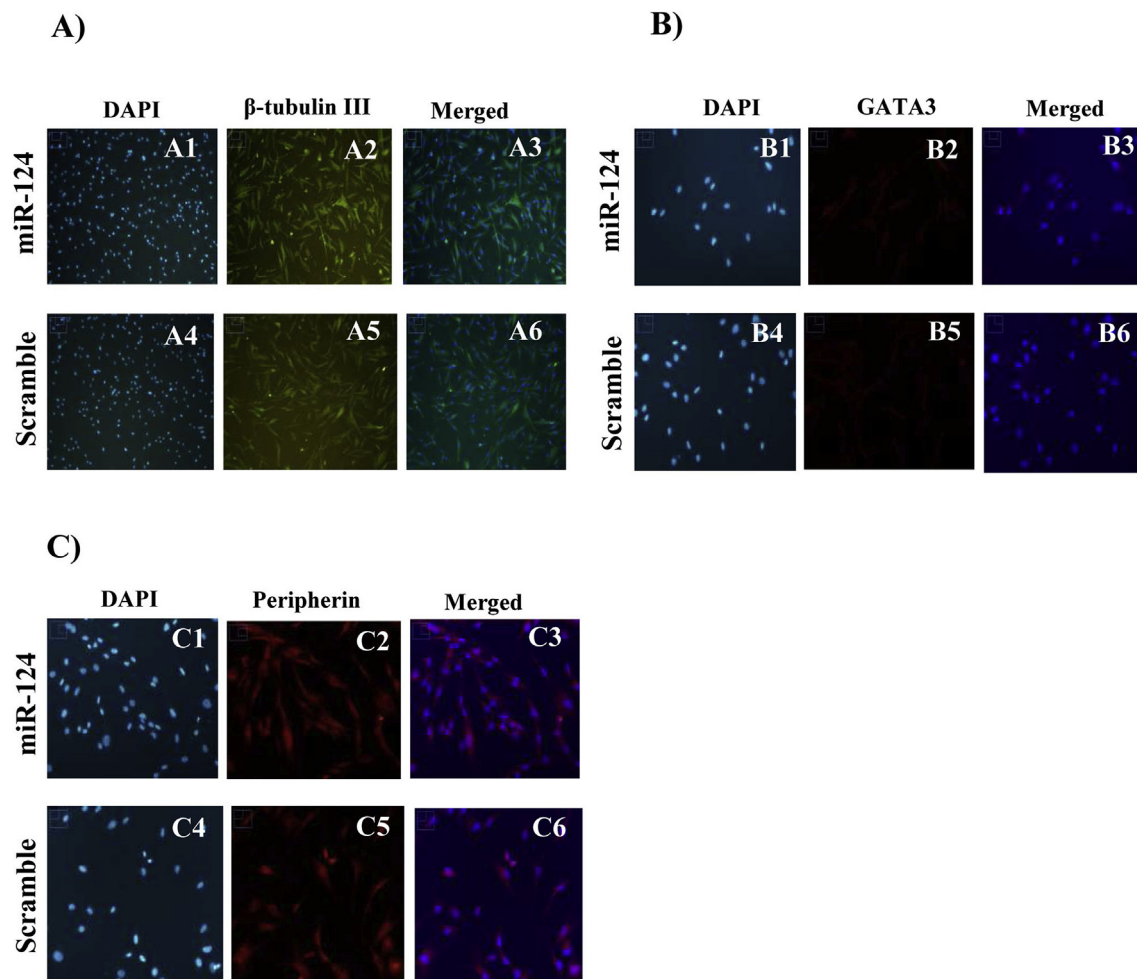




**Fig. 4.** qRT-PCR analyses of  $\beta$ -tubulin III, GATA-3, and peripherin mRNA expression in DPSCs post-transfection. The data show increased  $\beta$ -tubulin III mRNA expression 6 h (Mann–Whitney,  $P = 0.005$ ) and 16 h (Mann–Whitney,  $P = 0.008$ ) post-transfection as compared with that in the control (A). There was no statistically significant alteration in GATA-3 (B) and peripherin (C) mRNA expression in the scrambled control versus miR-124 transfected cells in any post-transfection hours.

transfected inner ear neural stem cells with miR-124 and observed alterations in the expression of TrkB and CDC42, thereby demonstrating a significant role for miR-124 in neuronal differentiation (Jiang et al., 2016). The results of the present study proposed that miR-124 promotes upregulation of Nestin and  $\beta$ -tubulin III in DPSCs but that it does not promote SGN differentiation or affect the phenotype. Similarly, a study conducted by Alonso et al. (Duran Alonso et al., 2012) showed that growth factors induced the differentiation of bone marrow-derived MSCs into Nestin- and SOX2-expressing neural progenitors (Duran Alonso et al., 2012). The results of the present study showed that increased levels of miR-124 did not lead to profound changes, as compared with those induced by growth factors. In this study, miR-124 influenced only the expression of Nestin, a neural progenitor marker. It is likely that the lack of significant effects induced by miR-124 is due to the concentration (24 nM) used. Future studies should examine the effects

of a range of concentrations of this miRNA. Although previous research has established the roles of various miRNAs in the progression of different cell processes, including development and differentiation (Elkan-Miller et al., 2011b; Friedman et al., 2009; Patel and Hu, 2012; Wang et al., 2010), miRNAs do not seem to be as effective as other factors (e.g., transcription factors and growth/neurotrophic factors) in increasing differentiation markers or inducing the morphology of interest in target cells. Thus, miRNAs should be used with other factors. The role of intracellular micro-environment required by miRNAs should not be forgotten either. As compared with the requirements of other factors, miRNAs may require further specific conditions to affect cells. On the other hand, miRNAs are more cost-effective than growth factors. Nevertheless, their unexpected outcomes should not be underestimated. Despite the well-established potential of DPSCs in neurogenesis, it is possible that the differentiate capacity of these cells into auditory



**Fig. 5.** Immunofluorescence analyses of  $\beta$ -tubulin III (A), GATA-3 (B), and peripherin (C) expression in DPSCs 6 h after transfection with miR-124 (A1,2,3; B1,2,3; C1,2,3) versus scrambled miR (A4,5,6; B4,5,6; C4,5,6). As shown by the figures, the immunofluorescence analysis revealed no significant alteration in the expression of  $\beta$ -tubulin III, GATA3 and Peripherin upon hDPSC transfection with miR-124 6 h post-transfection (however the results of qRT-PCR revealed upregulation of the neuron-specific marker  $\beta$ -tubulin III at the mRNA level).

neuron lines may be inadequate using a strategy involving miR-124.

## 5. Conclusion

The results indicated that transfection of DPSC with miR-124 mimic altered the expression of some neural progenitor and neural markers of SGNs in RNA or protein level. However, miR-124 did not significantly affect the expression of specific markers for SGNs.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

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